Weak acid and alkali stress regulate phosphatidylinositol bisphosphate synthesis in *Saccharomyces cerevisiae*

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Weak organic acids are used as food preservatives to inhibit the growth of spoilage yeasts, including *Saccharomyces cerevisiae*. Long-term adaptation to weak acids requires the increased expression of the ATP-binding cassette transporter Pdr12p, which catalyses the active efflux of the weak acids from the cytosol; however, very little is known about the signalling events immediately following application of weak acid stress. We have investigated the effects of weak acids on two stress-responsive signalling molecules, PtdIns(3,5)P_2 and PtdIns(4,5)P_2, which in *S. cerevisiae* are synthesized by Fab1p and Mss4p respectively. At low extracellular pH, benzoic acid, sorbic acid and acetic acid all cause a transient reduction in PtdIns(3,5)P_2 accumulation and a more persistent rise in PtdIns(4,5)P_2 levels. The increase in PtdIns(4,5)P_2 levels is accompanied by a reorganization of the actin cytoskeleton. However, changes in PtdInsP_2 levels are independent of weak acid-induced Pdr12p expression. In contrast, changing the extracellular medium to alkaline pH provokes a prolonged and substantial rise in PtdIns(3,5)P_2 levels. As PtdIns(3,5)P_2 synthesis is required for correct vacuole acidification, it is possible that levels of this molecule are modulated to maintain intracellular pH homoeostasis in response to weak acid and alkali stresses. In conclusion, we have expanded the repertoire of stress responses that affect PtdInsP_2 levels to include weak acid and alkali stresses.

Key words: alkali stress, Fab1p, Mss4p, *PDR12*, phosphatidylinositol bisphosphate, weak acid.

INTRODUCTION

Weak acids are widely used as food preservatives in order to inhibit the growth spoilage yeasts [1,2]. *Saccharomyces cerevisiae* is one of the common yeasts associated with large-scale food spoilage and acquires resistance to weak acids by increasing expression of the ABC (ATP-binding cassette) transporter, Pdr12p, which promotes the active efflux of these compounds [2–4]. Weak organic acids such as sorbic, benzoic and aliphatic short-chain (C-3 to C-8) carboxylic acids of reasonable water solubility all provoke increased expression of Pdr12p [2,4]; however, the timescale of Pdr12p expression and its mode of action suggest that this mechanism represents part of a long-term adaptation to weak acid stress. It is likely that yeast cells will also adopt short-term measures to maintain viability immediately following challenge with weak acids. The nature of any such short-term responses and the signalling events associated with them are yet to be characterized.

Phosphorylated derivatives of PtdIns, often referred to as PPIns (polyphosphoinositides), are a family of signalling molecules that function by binding effector proteins at cell membranes [5]. A network of kinases and phosphatases dynamically regulate PPIn levels, and activation/inhibition of these enzymes represents one of a number of mechanisms by which extracellular signals are transmitted to the cell's interior [6]. *S. cerevisiae* contain two PtdIns*P* kinases: Fab1p and Mss4p [7–9]. Fab1p synthesizes PtdIns(3,5)P_2 from PtdIns3*P* [9,10]. Genetic studies show that PtdIns(3,5)P_2 synthesis is required for a number of independently regulated processes including: vacuole acidification [11,12], growth at 37 °C [12], retrograde transport from the vacuole [13,14] and trafficking of some ubiquitinated cargoes to the vacuole lumen [15–18]. Mss4p is essential for viability, and is responsible for the production of PtdIns(4,5)P_2 from PtdIns4*P* [7,8]. Synthesis of PtdIns(4,5)P_2 is found to be required for an ever increasing number of processes [19], of which the best characterized in *S. cerevisiae* is regulation of the actin cytoskeleton [7,8,20]. Additionally, Mss4p also provides substrate for another signalling enzyme PLC (phospholipase C), Plc1p [7,8,20,21].

Different stresses influence the levels of PtdIns(3,5)P_2 and PtdIns(4,5)P_2 in *S. cerevisiae* cells. PtdIns(3,5)P_2 rapidly and transiently accumulates in response to hyper-osmotic stress [22], a response that is conserved in *Saccharomyces pombe*, plants and differentiated 3T3-L adipocytes [22–24]. Both PtdIns(3,5)P_2 and PtdIns(4,5)P_2 levels increase in response to heat stress [8] and PtdIns(4,5)P_2 levels decline rapidly on the application of a hypo-osmotic stress due to activation of PLC [21]. Additionally, recent studies have demonstrated that *mss4* and *fab1* mutants interact genetically with components of the heat shock response [20,25]. Thus, although it is unlikely that we have identified all the cellular processes requiring PtdIns(3,5)P_2 and PtdIns(4,5)P_2, it is clear that these molecules have highly conserved roles in many aspects of cell biology, and are likely to be pivotal in the commutation of signals generated by extracellular stresses. In this paper, we present results on the modulation and role of PtdInsP_2 in two additional stress responses: weak acid and alkali stress.

MATERIALS AND METHODS

Yeast strains, media and materials

All yeast strains used were purchased from EUROSCARF (Johann Wolfgang Goethe-University Frankfurt, Germany) and were derivatives of BY4741 (MATa; *his3*Δ*1*; *leu2*Δ*0*; *met15*Δ*0*; *ura3*Δ*0*). All chemicals unless otherwise stated were obtained from Sigma (St. Louis, MO, U.S.A.). [^{3}H]Inositol was from

Abbreviations used: ABC transporter, ATP-binding cassette transporter; MVB, multivesicular body; PLC, phospholipase C; PPIn, polyphosphoinositide; V-ATPase, vacuole ATPase.

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Amersham Biosciences (U.K.). Yeast strains were maintained on YEPD agar (pH 6.8) (1 % Difco yeast extract, 2 %, w/v, peptone, 2 %, w/v, glucose, 2 %, w/v, agar) or dropout agar medium [DO; 0.67 % yeast nitrogen base (Q-biogene), 2 % glucose and complete supplement mixture lacking the appropraite amino acids (Q-biogene)]. The YEPD (1 % yeast extract, 2 % peptone and 2 % glucose) or DO media (solid or liquid) was adjusted to pH 4.5 or pH 6.8 with either HCl or NaOH before autoclaving.

Analysis of Pdr12p levels and *PDR12-LacZ* expression

Measurement of the expression of cellular Pdr12p levels, and of *PDR12-LacZ* gene reporter of *PDR12* promoter activity were performed as described previously [4,26,27]. Briefly, to measure Pdr12p levels, cells were grown to mid-exponential phase and manipulated as outlined in the Figure legends, after which the cells were harvested, lysed and protein extracts were resolved by SDS/PAGE. We detected Pdr12p by Western blotting, and assayed total protein loading by blotting for Sba1p, a yeast chaperone whose expression levels are unaffected by weak acid stress.

To measure *PDR12* promoter activity, cells containing a *PDR12-LacZ* reporter gene under the control of the *PDR12* promoter were grown in YEPD to the mid-exponential growth phase. Cells were manipulated as outlined in the Figure legends, lysed and β-galactosidase activity was assayed.

In vivo phosphoinositide measurement

In vivo measurement of PPIn levels was performed as described previously [9,22,28]. Yeast cells were grown in synthetic complete supplementary medium [SD; 0.67 % (w/v) yeast nitrogen base without inositol (Q-biogene), 2 % glucose and 0.79 g of complete supplement mixture (Q-biogene)]. An overnight culture was diluted to 5×10^4 cells/ml and 10–20 μCi/ml [^{3}H]inositol was added. Cells were grown to a density of (2–4) $\times 10^6$ cells/ml (typically 12–16 h), after which the cells were manipulated as outlined in the relevant Figure legends, and killed by the addition of an equal volume of methanol. Yeast cells were harvested by centrifugation, disrupted by vortex-mixing with 0.4 g of glass beads (425–600 μm; Sigma) in the presence of 200 μl of methanol for 5 min and lipids were extracted and deacylated. The lipid head groups were analysed by HPLC, and peaks were detected by liquid-scintillation counting.

Phalloidin staining of yeast cells

Yeast cells were grown to mid-exponential in YEPD, challenged as indicated in the Figure legends, fixed by adding formaldehyde to a final concentration of 3.7 % (v/v) and incubated at 30 °C for 40 min. Cells were then washed twice in PBS (10 mM sodium phosphate, 2.7 mM potassium chloride and 137 mM sodium chloride; pH 7.4 at 25 °C), stained with 5 units of rhodamine–phalloidin per ml of initial culture volume at 30 °C for 1 h, washed three times with PBS and mounted on a polylysine-coated slides. Images were taken using a Delta Vision microscope (Applied Precision, Seattle, WA, U.S.A.) running SoftWoRx image capturing software.

RESULTS

Benzoic acid stress modulates *in vivo* PtdInsP_2 levels

We investigated the effects of weak acid stress on *in vivo* PPIn production by challenging yeast cells with 5 mM benzoic acid either in the presence or absence of 20 mM citrate (pH 4.5) as buffer (Figure 1). In an un-buffered medium, which typically has a pH of 5.0–5.5, benzoic acid provoked an approx. 3-fold accumulation in [^{3}H]PtdIns(3,5)P_2 levels (Figure 1A). Although hyperosmotic stress is able to stimulate [^{3}H]PtdIns(3,5)P_2 accumulation, addition of 5 mM benzoate (from a 0.5 M stock) will cause negligible osmotic stress [22] and thus the accumulation of [^{3}H]PtdIns(3,5)P_2 seen in these experiments is not due to an osmotic stress response. No changes in [^{3}H]PtdIns(4,5)P_2 levels were seen on addition of 5 mM benzoic acid to cells in an un-buffered medium (Figure 1B). Weak acid stress is enhanced at low pH [2,4], so we assayed PPIn levels in the presence of benzoate in a medium buffered to pH 4.5 with 20 mM citrate. Addition of 20 mM citrate (from a 400 mM stock) had no effects on PPIn levels; however, addition of 5 mM benzoic acid simultaneously with citrate buffer (20 mM sodium citrate buffered to pH 4.5 at 25 °C, and this was added directly to the yeast medium from a stock of 400 mM to give 20 mM final concentration) caused a significant decrease in [^{3}H]PtdIns(3,5)P_2 levels and an increase in [^{3}H]PtdIns(4,5)P_2 levels (Figures 1A and 1B). These effects of benzoic acid on PtdInsP_2 levels were identical in cells labelled for 12–16 h in a medium buffered to pH 4.5 with 20 mM citrate, and in cells labelled in an un-buffered medium to which the citrate buffer (pH 4.5) and benzoic acid were added simultaneously (results not shown), indicating that these effects were a result of the low extracellular pH rather than the citrate. Whilst these results were initially confusing, further investigation revealed that addition of the preparation of benzoic acid (actually sodium benzoate) used in these experiments to an un-buffered SD medium caused a rise in pH, typically from pH 5.0–5.5 to pH 8.0–8.5. Thus the effects

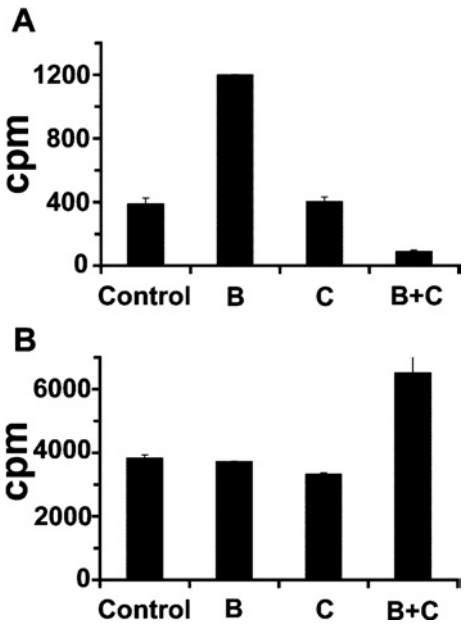

Figure 1 Effects of challenging yeast cells with 5 mM benzoate on PtdInsP_2 levels

Yeast cells were labelled with [^{3}H]inositol in an un-buffered inositol-free medium. Aliquots (1.8 ml) were challenged with 5 mM benzoic acid (B), 20 mM citric acid (pH 4.5) (C), or benzoic acid and citric acid (B + C) as indicated, for 10 min. Lipids were extracted and processed as described in the Materials and methods section. All data are presented as c.p.m. and error bars represent the range of the data ($n = 2$). The results presented are typical of one of three experiments. No significant changes were seen in [^{3}H]PtdIns3*P* and [^{3}H]PtdIns4*P* levels (results not shown). (**A**) [^{3}H]PtdIns(3,5)P_2 levels are modulated by benzoic acid stress. Addition of benzoic acid to an un-buffered medium provokes an accumulation of [^{3}H]PtdIns(3,5)P_2. Addition of 20 mM citric acid (pH 4.5) (C) has no effect; however, simultaneous application of benzoic acid and citric acid (B + C) causes a reduction in [^{3}H]PtdIns(3,5)P_2 levels. (**B**) [^{3}H]PtdIns(4,5)P_2 levels are modulated by benzoic acid stress. Application of benzoic acid or citric acid to un-buffered cells had no effect on [^{3}H]PtdIns(4,5)P_2 levels; however, simultaneous application of both acids provoked an increase in [^{3}H]PtdIns(4,5)P_2 levels.

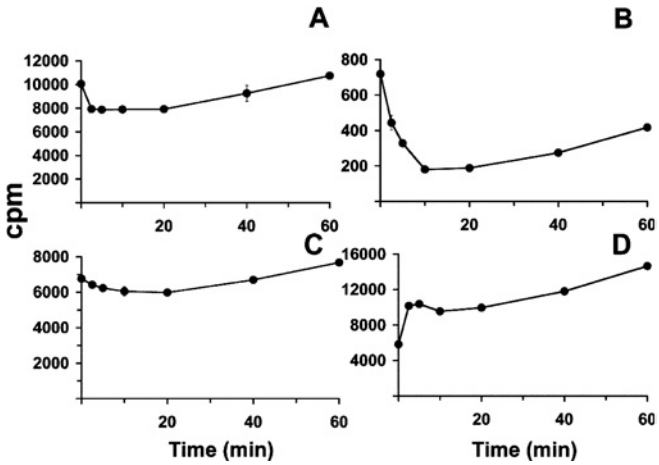

Figure 2 Effects of benzoic acid on PPIn levels

Yeast cells were labelled with [^{3}H]inositol in an inositol-free medium buffered to pH 4.5 with 20 mM citrate. Aliquots (1.8 ml) of cells were challenged with 5 mM benzoic acid for the times indicated (min). Lipids were extracted and processed as described in the Materials and methods section. All data are given as c.p.m. and error bars represent the range of the data ($n = 2$). The graphs represent the levels of [^{3}H]PtdIns3*P* (**A**), [^{3}H]PtdIns(3,5)P_2 (**B**), [^{3}H]PtdIns4*P* (**C**) and [^{3}H]PtdIns(4,5)P_2 (**D**). Benzoic acid provoked a small loss in [^{3}H]PtdIns over the time course of the experiment (251 000 c.p.m. at 0 min to 197 000 c.p.m. at 60 min); however, this does not account for the changes seen in the other PPIns.

of benzoic acid on PPIn levels we observe in an un-buffered medium could be due to the rise in pH, or a combined effect of the rise in pH and simultaneous challenge with benzoic acid. In light of these data, we investigated the effects of both weak acid and pH change on PPIn levels.

Effects of benzoic acid on PPIn levels

We investigated the effects of 5 mM benzoic acid at pH 4.5 on PPIn levels for up to 60 min after challenge (Figure 2). [^{3}H]PtdIns(3,5)P_2 levels rapidly decline on application of 5 mM benzoic acid, reaching approx. 25 % of un-stressed levels after 10–15 min (Figure 2B), after which [^{3}H]PtdIns(3,5)P_2 begins to recover back towards resting levels over the course of the experiment (60 min), a pattern of change indicative of an adaptive response. In contrast, [^{3}H]PtdIns(4,5)P_2 levels rise rapidly to approx. 2-fold of un-stressed levels 2–3 min after challenge, decline slightly and then continue to rise gradually over the duration of the experiment (Figure 2D). There are only very low transient decreases in both [^{3}H]PtdIns3*P* and [^{3}H]PtdIns4*P* levels (Figures 2A and 2C).

Other organic weak acids provoke similar changes in [^{3}H]PtdInsP_2 accumulation

Benzoic acid is only one of a number of weak organic acids that mediate a stress response [4]. To investigate whether the effects we have seen on [^{3}H]PtdInsP_2 synthesis are specific for benzoic acid or are part of a general response to weak acid stress, we compared the effects of benzoic acid with sorbic acid and acetic acid. We also included phenylalanine as a control for osmotic effects. Like benzoic acid, challenge with sorbic acid and acetic acid all cause a reduction in [^{3}H]PtdIns(3,5)P_2 and an increase in [^{3}H]PtdIns(4,5)P_2 levels (Figures 3A and 3B), with only small changes in [^{3}H]PtdIns3*P* and [^{3}H]PtdIns4*P* levels (results not shown). In contrast, phenylalanine had no effects on the levels of any [^{3}H]PPIn. Thus the changes in [^{3}H]PtdInsP_2 levels observed in our experiments are likely to be part of a general stress response to weak acids.

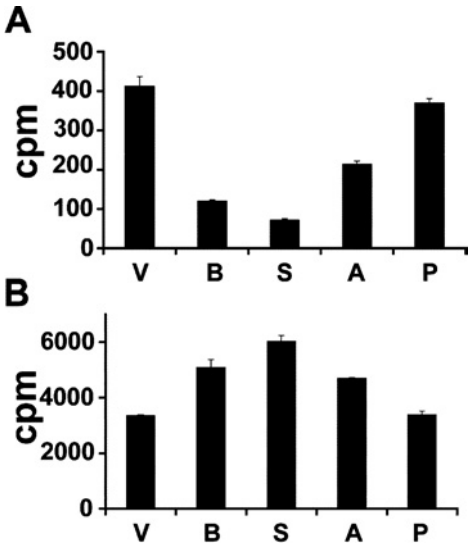

Figure 3 Changes in PtdInsP_2 levels in response to weak acids

Yeast cells were labelled with [^{3}H]inositol in inositol-free SD medium, and 1.8 ml aliquots were challenged with vehicle (distilled water; V), 5 mM benzoic acid (B), 5 mM sorbic acid (S), 5 mM acetic acid (A) or 5 mM phenylalanine (P) as indicated for 12 min in a medium buffered to pH 4.5 with 20 mM citric acid. PPIns were analysed as described in the Materials and methods section. All data are given as c.p.m. and error bars represent the range of the data ($n = 2$). The results presented are typical of at least three similar experiments. (**A**) [^{3}H]PtdIns(3,5)P_2 levels are reduced by weak acid stress. All weak acids provoked a decline in [^{3}H]PtdIns(3,5)P_2 levels when compared with vehicle or phenylalanine. (**B**) [^{3}H]PtdIns(4,5)P_2 levels increase in response to weak acid stress. All weak acids provoked an increase in [^{3}H]PtdIns(4,5)P_2 levels when compared with vehicle or phenylalanine. These results indicate that these changes in PtdInsP_2 levels are a response to general weak acid stress and not specific to benzoic acid. No changes in [^{3}H]PtdIns levels were seen between vehicle and control (results not shown).

Sorbic acid or benzoic acid induction of Pdr12p expression does not require PtdIns(3,5)P_2

Challenging *S. cerevisiae* with sorbic acid or benzoic acid increases Pdr12p expression [4,26]. To test if PtdIns(3,5)P_2 has any role in this response, we measured benzoic acid- and sorbic acid-mediated Pdr12p expression in wild-type and *fab1*Δ cells – *fab1*Δ cells cannot synthesize PtdIns(3,5)P_2 [9] – using two independent methods: Western blotting for native Pdr12p, and assaying *LacZ* reporter gene expression from the *PDR12* promoter (Figures 4A and 4B). As a control, we again used 5 mM phenylalanine. There are no differences between wild-type and *fab1*Δ cells in Pdr12p expression on challenge with weak acids (Figures 4A and 4B). Surprisingly, challenge with 5 mM phenylalanine also provokes an increase in both Pdr12p levels, and *LacZ* reporter activity, an observation that has not previously been reported. *pdr12*Δ cells are extremely sensitive to growth in the presence of weak acids [2], so we investigated how weak acids affect growth of *fab1*Δ cells (Figure 4C); *fab1*Δ cells are neither more nor less resistant to growth in the presence of weak acids than wild-type cells. As phenylalanine promotes increased Pdr12p expression without affecting PtdInsP_2 levels, it is likely that PtdInsP_2 has no role in induction of Pdr12p expression.

Weak acid stress causes changes in the actin cytoskeleton

PtdIns(4,5)P_2 is required for many stages in regulation of the actin cytoskeleton [7,8,20,29], and thus the increase in PtdIns(4,5)P_2 synthesis seen on challenge with weak acids might be associated with changes in the actin cytoskeleton (Figure 5). In unchallenged

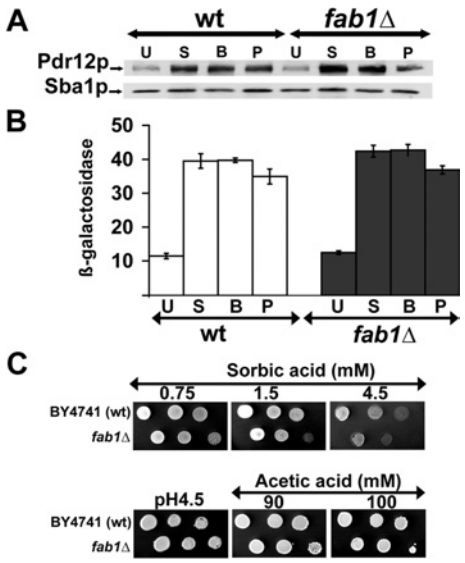

Figure 4 Comparison of responses to weak acid in wild-type and *fab1*Δ cells

(**A**) Immunoblot detection of endogenous weak acid stress-induced Pdr12p levels in wild-type (wt) and *fab1*Δ cells. Cells were grown to mid-exponential phase and challenged with 5 mM sorbate (S), benzoate (B) or phenylalanine (P) at pH 4.5 for 2 h as indicated. Pdr12p levels were analysed by Western blotting. Un-stressed cells (U) are shown as a control. Acetate stress was not used in this assay as 5 mM acetate has already been shown not to induce Pdr12p [4]. Levels of Sba1p, a yeast co-chaperone, were also measured as a control for total protein loading. *fab1*Δ cells show similar induction of Pdr12p in response to weak acid stress as wild-type cells. (**B**) Expression of episomal *PDR12* promoter-*LacZ* reporter activity in the wild-type cells and the *fab1*Δ mutant. Cells were grown to mid-exponential phase in pH 4.5 and challenged for 2 h with 5 mM sorbate (S), benzoate (B) or phenylalanine (P) as indicated. Cells were killed, and LacZ activity was measured as described in the Materials and methods section. Activity is given as arbitrary units and error bars represent the S.E.M. ($n = 3$). (**C**) Growth of wild-type and *fab1*Δ cells in the presence of sorbate or acetate at pH 4.5. Serial dilutions of wild-type and *fab1*Δ cells were plated on plates containing various concentrations of weak acids as indicated. Plates were photographed after 4 days of incubation at 24 °C. *fab1*Δ cells are neither more nor less sensitive to weak acid stress when compared with wild-type cells.

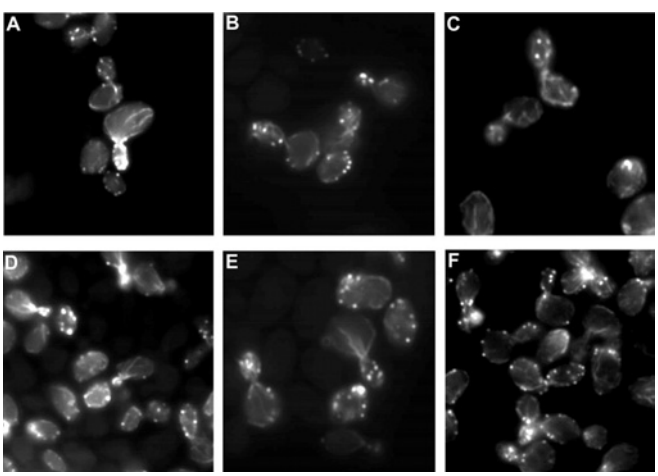

Figure 5 The actin cytoskeleton changes in response to weak acid stress

Yeast cells were challenged with 5 mM sorbic acid for 5 min (**B**), 10 min (**C**), 20 min (**D**) or 60 min (**E**), fixed and stained with rhodamine–phalloidin as described in the Materials and methods section. Un-stressed cells (**A**) and cells challenged with 1 M sorbitol for 14 min (**F**) are shown for comparison. Addition of weak acids to cells after fixing had no effects on actin staining (results not shown).

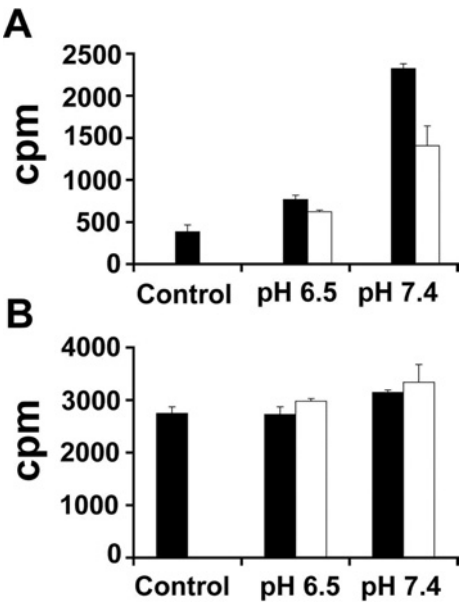

Figure 6 Alkaline stress activates [^{3}H]PtdIns(3,5)P_2 synthesis

Aliquots (1.8 ml) of yeast cells, labelled in un-buffered inositol-free SD medium, were challenged with 50 mM Tris, pH 6.8 (to final pH 6.5) or pH 7.6 (final pH 7.4), and either vehicle (solid bars) or 5 mM benzoic acid (open bars) for 10 min. [^{3}H]PtdInsP_2 levels were measured as described in the Materials and methods section. Data are presented as c.p.m. and error bars represent the range of the data ($n = 2$). The results presented are representative of at least four similar experiments. (**A**) [^{3}H]PtdIns(3,5)P_2 levels increase approx. 6-fold on changing the extracellular pH to 7.4, but only rise less on increasing the extracellular pH to 6.5 when compared with control levels. Simultaneous addition of 5 mM benzoic acid causes a smaller decrease in [^{3}H]PtdIns(3,5)P_2 levels at both pH values when compared with buffer alone. (**B**) Increase in extracellular pH had no effect on [^{3}H]PtdIns(4,5)P_2 levels. There were no significant changes in the levels of [^{3}H]PtdIns3*P* and [^{3}H]PtdIns4*P* (results not shown).

cells, most of the cortical actin deposition is in the daughter cell, and actin stress fibres run from the budding daughter cell through the mother cell. Following challenge with 5 mM sorbic acid, cells lose actin stress fibres (Figure 5B) and there is an increase in cortical actin deposition in the mother cell. Cortical actin deposition is greatly reduced 10 min after challenge (Figure 5C). By 60 min, stress fibres have returned to the cells; however, there appears to be an increase in cortical actin patches in the mother cell (Figure 5E). For comparison, we have shown the enhanced cortical actin deposition due to a hyper-osmotic stress (Figure 5F) [30].

Alkali stress activates [^{3}H]PtdIns(3,5)P_2 synthesis

Our initial experiments with benzoic acid using an un-buffered medium suggested that raising extracellular pH could stimulate increased [^{3}H]PtdIns(3,5)P_2 synthesis. To test this, we challenged yeast labelled in an un-buffered medium (typically pH 5.0–5.5) with 50 mM Tris, pH 6.8 or pH 7.6 (diluted from a 0.5 M stock to give a final pH of 6.5 and 7.4 respectively), in the presence and absence of 5 mM benzoic acid (Figure 6). Whilst pH shift to 6.5 provoked only a small rise in [^{3}H]PtdIns(3,5)P_2 levels, shift to 7.4 caused a 6-fold rise (Figure 6A). Simultaneous addition of 5 mM benzoic acid with the Tris buffer (50 mM Tris at pH either 6.8 or 7.5 at 25 °C, and this was added directly to the yeast medium from a 0.5 M stock to give 50 mM final concentration) caused the pH-induced level of [^{3}H]PtdIns(3,5)P_2 to be reduced slightly (Figure 6A), confirming that our original observations using 5 mM benzoic acid in an un-buffered medium (Figure 1A) were due to increase in extracellular pH. Addition of 50 mM Tris to

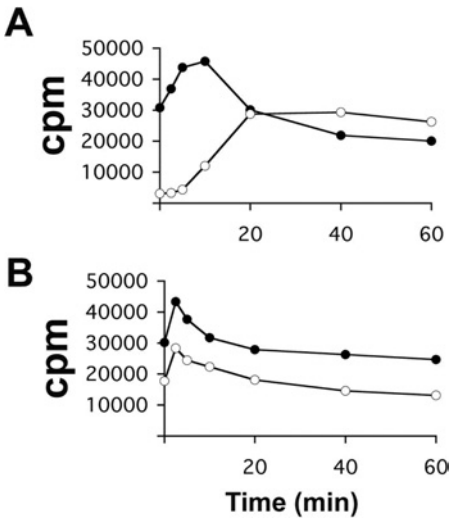

Figure 7 Effects of alkali stress on PPIn levels

Aliquots (1.8 ml) of yeast, labelled with [^{3}H]inositol in an un-buffered medium, were challenged with 50 mM Tris, pH 7.6 (final pH 7.4), for the times indicated (min). Lipid levels were analysed as described in the Materials and methods section. Data are given as c.p.m. and error bars represent the range of the data ($n = 2$). (**A**) [^{3}H]PtdIns3*P* (●) and [^{3}H]PtdIns(3,5)P_2 (○) levels in response to alkaline pH stress. Application of 50 mM (final concentration) Tris (pH 7.5) causes a small rise in [^{3}H]PtdIns3*P* levels followed by a gradual decline, consistent with an increase in [^{3}H]PtdIns(3,5)P_2 synthesis. [^{3}H]PtdIns(3,5)P_2 levels start to increase 5 min after stimulation and reach a plateau after 20 min at a level approx. 10-fold over control levels. (**B**) [^{3}H]PtdIns4*P* and [^{3}H]PtdIns(4,5)P_2 levels in response to alkaline pH stress. Both [^{3}H]PtdIns4*P* (●) and [^{3}H]PtdIns(4,5)P_2 (○) levels show a low spike reaching a peak at 2.5 min, before returning to control levels for the duration of the experiment.

yeast medium would not cause a large enough osmotic shock to stimulate significant [^{3}H]PtdIns(3,5)P_2 production [22]. No significant changes were seen in [^{3}H]PtdIns(4,5)P_2 levels (Figure 6B), nor in [^{3}H]PtdIns3*P* and [^{3}H]PtdIns4*P* levels (results not shown) at either pH.

Effects of alkali stress on [^{3}H]PPIn levels

We investigated the effects of alkali stress on PPIn levels over time by challenging yeast labelled in an un-buffered medium with 50 mM Tris (pH 7.6) for up to 60 min (Figure 6). [^{3}H]PtdIns3*P* levels rise rapidly on application of pH stress, reaching a peak after 10 min and then declining, consistent with [^{3}H]PtdIns3*P* being consumed by increased [^{3}H]PtdIns(3,5)P_2 synthesis. [^{3}H]PtdIns(3,5)P_2 levels start to rise 5 min after stress, reaching a peak at approx. 20 min and then maintaining this elevated level for the duration of the experiment (Figure 7A). The dynamics of this response are markedly different from those of the osmotic stress response, where there is significant accumulation of [^{3}H]PtdIns(3,5)P_2 within 1 min following application of stress and [^{3}H]PtdIns(3,5)P_2 returns to un-stressed levels after approx. 60 min [22]. [^{3}H]PtdIns(4,5)P_2, as well as its precursor [^{3}H]PtdIns4*P*, shows a low spike in levels approx. 2.5 min after application of alkali stress (Figure 7B). Alkali stress has been shown to cause a spike in intracellular calcium [31], and as Mss4p can be activated by calmodulin [32], this could account for the spike in [^{3}H]PtdIns(4,5)P_2 levels. However, whether these changes have any role in alkali stress responses remains to be determined.

*fab1*Δ cells are sensitive to alkali stress

It has previously been reported that the growth of *fab1*Δ cells is sensitive to high pH [33]. We further characterized this by comparing the growth of *fab1*Δ cells with that of wild-type and *vma6*Δ cells – Vma6p is a component of the V-ATPase (vacuole ATPase) that is required for correct vacuole acidification [34] – on medium buffered to pH 7.6 or 8.0. *vma6*Δ cells are unable to grow at either pH 7.6 or 8.0; however, *fab1*Δ cells could grow at pH 7.6, but were compromised at pH 8.0 (Figure 8). This result is broadly in agreement with separate studies on alkali pH-sensitive mutants [33,35] and would imply that the vacuole acidification defect of *fab1*Δ cells is less severe than that of *vam6*Δ cells.

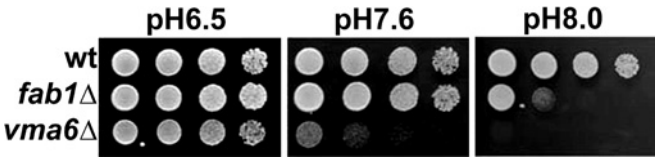

Figure 8 Comparison of the effects of pH on growth of wild-type, *fab1*Δ and *vma*Δ*6*Δ cells

Mid-exponential cultures of yeast cells were diluted to 10^7 cells/ml and serial 5-fold dilutions were made; 10 μl of each dilution was spotted on to YEPD, or YEPD adjusted to pH 7.6 or 8.0 and buffered with 50 mM Tris as indicated. Plates were incubated for 4–6 days at 24 °C. Wild-type (wt) cells were able to grow at all pH values; however, *fab1*Δ cell growth was sensitive to pH 8.0, and *vam6*Δ cell growth was sensitive to pH 7.6 and 8.0.

DISCUSSION

In *S. cerevisiae*, levels of the inositol lipid second messengers PtdIns(3,5)P_2 and PtdIns(4,5)P_2 are modulated in response to a variety of stresses including heat, hypo-osmotic and hyper-osmotic stresses [8,21,22], and both *FAB1* and *MSS4*, which code for the enzymes responsible for PtdIns(3,5)P_2 and PtdIns(4,5)P_2 synthesis respectively, interact genetically with elements of the heat-shock response pathway [20,25]. The results presented herein have widened the repertoire of stress responses that affect PtdInsP_2 levels to include both weak acid and alkali stresses.

Role of PtdInsP_2 in Pdr12p expression

At low extracellular pH, PtdIns(3,5)P_2 levels decrease transiently in response to challenge with weak acids; in contrast, PtdIns(4,5)P_2 levels increase 2-fold following weak acid stress and are elevated for at least 60 min. In *S. cerevisiae*, the canonical response to weak acid stress is the increased expression of the ABC transporter Pdr12p that is responsible for the removal of intracellular weak acids [2]. Our results show that stress-induced changes in PtdInsP_2 levels are independent of increased Pdr12p expression for the following reasons: firstly, *fab1*Δ cells, which cannot synthesize PtdIns(3,5)P_2 [10,36], show no obvious deficiencies in weak acid-induced Pdr12p expression; secondly, 5 mM acetic acid provokes changes in PtdInsP_2 levels but does not affect Pdr12p expression [2,4]; and finally, challenging yeast cells with 5 mM phenylalanine, which provokes no changes in PtdInsP_2 levels, causes increased Pdr12p expression. Phenylalanine has thus proved to be a fortuitous control in our experiments. At low pH, weak organic acids are un-dissociated and predominantly enter yeast cells by passive diffusion across the plasma membrane, resulting in acidification of the cell's interior [2,37]. Phenylalanine cannot enter the cell by passive diffusion, and will not directly affect intracellular pH; however, as weak acid-induced Pdr12p expression does not seem to be mediated via changes in intracellular pH [2], it is probable that weak acids and phenylalanine cause increased Pdr12p expression by a common mechanism that is independent of PtdInsP_2 synthesis. It has been proposed that weak acids activate transcription of *PDR12* by binding directly to

the transcription factor War1p, a mechanism that would render a second messenger unnecessary [2,26]. Thus the changes in PtdInsP_2 levels reported in the present study represent a novel response to weak acid stress, and could well be part of short-term defence measures taken by yeast cells.

PtdIns(4,5)P_2 in weak acid stress

PtdIns(4,5)P_2 is required for many steps in actin polymerization/organization [7,8,20,29]. The increase in PtdIns(4,5)P_2 levels seen in response to weak acid stress is accompanied by changes in the actin cytoskeleton. On application of weak acid stress, there is an initial loss of actin stress fibres, which recover after approx. 20 min. Cortical actin deposition is initially enhanced in the mother cell, and then is substantially reduced after 10 min of stress. At more prolonged time points, there is again enhanced deposition of cortical actin. This biphasic pattern of cortical actin deposition is paralleled by the changes seen in PtdIns(4,5)P_2 following weak acid stress. Furthermore, the enhanced deposition of cortical actin is similar to that seen in response to hyperosmotic stress [30,38]. Like weak acid stress, challenging yeast cells grown in YEPD with a hyperosmotic stress also causes a sustained increase in PtdIns(4,5)P_2 (F. T. Cooke, unpublished work), and we feel it is likely that the actin rearrangements seen in response to both stresses are related to enhanced PtdIns(4,5)P_2 production. Furthermore, the previously reported translocation of the inositol phosphate 5-phosphatases Inp52p and Inp53 to these actin patches would also imply a role for PtdIns(4,5)P_2 in their formation [38]. How might a more formal link between PtdIns(4,5)P_2 production and actin reorganization be demonstrated? *MSS4* is an essential gene, and it is possible that its role in weak acid stress could be addressed using conditional mutants; however, heat shock also activates PtdIns(4,5)P_2 synthesis [8], which might confound interpretation of these experiments. A more fruitful approach might be to try to identify effectors/activators of Mss4p via more classical genetic means; for example, *slm4* (YBR007c) mutants were independently identified in screens for synthetic lethality with *mss4* mutants [20] and hypersensitivity to weak acid stress [39], making Slm4p a potential candidate activator/effector of Mss4p-mediated stress signalling. The mechanisms by which weak acid stress activates PtdIns(4,5)P_2 synthesis are also unknown. Weak acids are unlikely to act directly on Mss4p, as we find no effects of weak acids on recombinant Mss4p activity *in vitro* (results not shown). The known components upstream of Mss4p, such as Rom2p, Rho1p and Tor2p, would be good candidates for further study [8,40].

PtdIns(3,5)P_2 in weak acid and alkali stress

Despite a transient decrease in PtdIns(3,5)P_2 levels in response to challenge with weak acids, the role of PtdIns(3,5)P_2 in weak acid stress is equivocal as growth of *fab1*Δ cells is neither more nor less sensitive to the presence of weak acids, and cells show a wild-type induction of Pdr12p. This of course does not mean that Fab1p has no role in weak acid stress, just that the cellular processes regulated by PtdIns(3,5)P_2 are not necessary for surviving challenge with weak acids. In contrast, alkali stress provokes a prolonged accumulation in PtdIns(3,5)P_2 levels, and, as previously reported, we have found that *fab1*Δ cell growth is sensitive to alkali pH, as would be expected of cells that fail to acidify their vacuoles correctly [33,35]. Previous work shows that PtdIns(3,5)P_2 synthesis is required for correct acidification of the vacuole [12,41] and PtdIns(3,5)P_2 could well be a key regulator of vacuole/endocytic pH. How this might be mediated is unknown as none of the PtdIns(3,5)P_2 effector proteins described to date are needed for vacuole acidification [14,42–44]. Nevertheless, generation of PtdIns(3,5)P_2 must increase net entry of H^+ into the vacuole/endosome, and potential mechanisms for this include activation of the V-ATPase complex, or modulation of an ion channel, such as a Cl^- channel. Our results and results of others [31,33,35] are consistent with PtdIns(3,5)P_2 production being either upstream or independent of V-ATPase activity, because if PtdIns(3,5)P_2 activates the V-ATPase, it could be imagined that in the absence of PtdIns(3,5)P_2 there could still be a very low level of tonic V-ATPase activity. In contrast, loss of V-ATPase subunits would completely ablate V-ATPase activity. This could explain why *vma6*Δ cells have a more severe vacuole acidification defect than *fab1*Δ cells, and their growth is more sensitive to alkali pH [33,35]. This matter will probably only be resolved after the PtdIns(3,5)P_2 effectors required for vacuole acidification are identified.

How might PtdIns(3,5)P_2-mediated regulation of vacuole/endocytic H^+ flux be relevant to weak acid and alkali stresses? At low extracellular pH, un-dissociated organic weak acids cross the plasma membrane by passive diffusion and acidify the cell interior causing a decrease in both cytosolic and vacuole pH [2,37]. Under these circumstances, yeast cells would need to expel H^+ ions, and do so, at least in part, by increasing the activity of the plasma membrane H^+-ATPase Pma1p [37,45,46]. As both the pH of the cytosol and the pH of the vacuole are reduced by weak acids, pumping cytosolic H^+ ions into the vacuole would be counter-productive to a cell trying to expel H^+ ions; thus PtdIns(3,5)P_2 synthesis could be reduced to prevent further acidification of the vacuole. Conversely, alkali stress causes an increase in the pH of the vacuole, probably via the uptake of extracellular medium by fluid phase endocytosis: it is known that increasing extracellular pH mimics the trafficking defects caused by loss of V-ATPase activity [47], demonstrating that increasing extracellular pH does indeed de-acidify the vacuole. Under these circumstances, PtdIns(3,5)P_2 synthesis could be activated to promote re-acidification of the vacuole/endosome. The dynamics of the PtdIns(3,5)P_2 response to alkali stress are consistent with this hypothesis. Application of alkali stress does not cause an immediate increase in PtdIns(3,5)P_2 synthesis; PtdIns(3,5)P_2 levels only start to rise 5 min after the application of stress, and this lag could be due to the time it takes for enough extracellular medium to transit to the vacuole/late endosome to cause de-acidification. Furthermore, the persistent increase in PtdIns(3,5)P_2 in response to alkali stress would also be consistent with the relatively large reservoir of extracellular buffer used in our experiments.

PtdIns(3,5)P_2, and perhaps PtdIns3*P*, could also have some role in the processing of the transcription factor RIM101, which mediates the transcriptional response to alkali stress in *S. cerevisiae*. It is now clear that correct activation of RIM101 requires an intact MVB (multivesicular body) trafficking pathway [48,49]; this pathway mediates the targeting of proteins to the yeast vacuole and Fab1p has been implicated in it [15–17]. Although the exact role of PtdIns(3,5)P_2 in MVB trafficking remains equivocal, it is possible that the changes in PtdIns(3,5)P_2 and PtdIns3*P* seen in response to alkali stress are part of the mechanism by which RIM101 becomes activated. However, it is unlikely that Fab1p's only role in alkali stress is to ensure activation of RIM101, as growth of *rim101* mutants, unlike *fab1* mutants, is not sensitive to alkali pH [33].

Conclusions

PtdIns(3,5)P_2 and PtdIns(4,5)P_2 are important regulators of numerous cell functions, and it is probable that these molecules have an important role in the commutation of a wide variety of stress signals in *S. cerevisiae*. We have now described two new cell

stresses that mediate changes in PtdInsP_2: weak acid stress and alkali stress. We think it is likely that PtdIns(4,5)P_2 mediates changes in the actin cytoskeleton in response to weak acid stress, whilst PtdIns(3,5)P_2 levels are modulated in weak acid and alkali stress to try to maintain correct pH balance across intracellular membranes. The elucidation of the mechanisms by which PtdInsP_2 levels are regulated and which cellular events are subsequently affected in response to stress will be of significance in developing our understanding of the molecular mechanisms underpinning stress responses.

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